# XENOBIOTIC RECEPTOR PXR-MEDIATED RESISTANCE TO *SALMONELLA* INVASION AND ITS MECHANISM

A Thesis

by

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ABSTRACT

Pregnane X receptor (PXR) is a ligand-activated nuclear receptor that regulates gene expression of the metabolizing enzymes that are involved in detoxification of endogenous and exogenous compounds in the gastrointestinal (GI) tract. In recent years, PXR has been found to have novel functions in maintenance of homeostasis of the GI tract, as mice deficient in PXR have spontaneous intestinal inflammation. Because the GI tract homeostasis is maintained through interactions between microbiota and host intestinal cells, we investigated the role of PXR in regulating the susceptibility of the colon cells to *Salmonella* infection. Using PXR-transfected HCT116 and HT29 cell lines we found that PXR transfected cells were resistant to the *Salmonella* infection and the resistance was dependent on the PXR activation by the typical PXR agonists rifampicin (RIF) and hyperforin. Furthermore, the ligand-dependent resistance could be antagonized by the PXR antagonist ketoconazole, confirming the role of PXR in resistance. In an earlier study, we found NF-κB activation by LPS to inhibit PXR activity, and, consistent with the observation, LPS treatment of the cells weakened the resistance of the PXR-transfected cells to *Salmonella* infection. To understand the mechanism of the PXR-regulated resistance, we analyzed the role of PXR in regulating the intestinal barrier gene expressions such as occludin, ZO-1, and claudin, and found that occludin expression was significantly up-regulated by the PXR activation. Taken together, our results suggest that PXR plays a role in regulating GI tract resistance to

pathogenic bacterial infection and provides a potential therapeutic approach for treatment of *Salmonella* infection.

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# NOMENCLATURE

| | |
|---|---|
| PXR | Pregnane X Receptor |
| CAR | Constitutive Androstane Receptor |
| IBD | Inflammatory Bowel Disease |
| ADI | Adverse Drug Interaction |
| AhR | Aryl Hydrocarbon Receptor |
| GR | Glucocorticoid Receptor |
| HR | Hormone Receptor |
| RAR | Retinoid Acid Receptor |
| VDR | Vitamin D Receptor |
| DBD | DNA Binding Domain |
| LBD | Ligand Binding Domain |
| ER | Estrogen Receptor |
| LXR | Liver X Receptor |
| ER | Estrogen Receptor |
| RXRα | Retinoid X Receptor Alpha |
| SHP | Short Heterodimer Partner |
| PR | Progesterone Receptor |
| DME | Drug Metabolizing Enzyme |
| SJW | St John's Wort |
| DDI | Drug-Drug Interaction |

| | |
|---|---|
| PAH | Polycyclic Aromatic Hydrocarbon |
| BaP | Benzo[*a*]pyrene |
| LCA | Lithocholic Acid |
| RIF | Rifampicin |
| TCM | Traditional Chinese Medicine |
| XREM | Xenobiotic Response Element |
| LPS | Lipopolysaccharide |
| TLR | Toll-like Receptor |
| IPA | 3-propionic Acid |
| T3SS | Type 3 Secretion System |
| SPI-1 | *Salmonella* Pathogenicity Island-1 |
| FBS | Fetal Bovine Serum |
| LB | Luria–Bertani |
| PBS | Phosphate-buffered Saline |
| CFU | Colony Forming Unit |

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# 1. INTRODUCTION

The interaction between gene and environment is an important force continuously governing the genotypes and phenotypes of organisms. To deal with the unavoidable exposure to the xenobiotics in surrounding environment, animals have developed an efficient defense system consisted of the so-called xenobiotic receptors, such as pregnane X receptor (PXR, also known as the steroid and xenobiotic receptor, SXR) and constitutive androstane receptor (CAR). These become parts of the "chemical defense"; If faced with infectious agents, organisms display innate and adaptive immune response, that the nuclear factor-κB (NF-κB) is the most important regulator in it. Collectively, these consist the parts of the “biological defense” mechanisms. These two arms of the defense systems are critical for the survival and evolutionary success of the organism. In recent years, increasing evidence suggests that these two systems are integrated to govern the wellbeing of the organisms and their crosstalk is found the to be the underpinning mechanism for some diseases such as the inflammatory bowel disease (IBD), adverse drug-drug interactions (ADIs). Modulation of the crosstalk through therapeutic drugs has been shown to be effective in treating the IBD. My research explores the role of PXR in controlling the mechanism of defense against the bacterial infections in cell culture model.

## 1.1 Xenobiotic receptors and their roles in metabolic detoxification

Xenobiotics (foreign compounds) are generally referred to as the compounds or substances that are normally not present in the organisms. The xenobiotic compounds

may be produced synthetically or generated naturally such as the plant and microbial metabolites, animal excretions, environmental pollutants, pesticides, industrial chemicals, and therapeutic drugs. They are an unavoidable part of our everyday life.

In order to survive, the organisms need to interact with their environment and this requires an efficient system to deal with the harmful xenobiotics. In animals, this defense network consists of the xenobiotic detoxification system. This system includes the phase I and phase II metabolizing enzymes, which PXR is involved in by regulating some important enzymes, such as cytochrome P450 (CYP) 3A4.

Phase I reactions are catalyzed by the monooxygnase enzymes such as cytochromes P450 that modified the molecules to be more polar and more susceptible to phase II reactions. Phase II conjugating reactions conjugated xenobiotics with charged species such as glutathione (GSH). Phase II reactions are to increase molecular weight and add large anionic groups (GSH) that they cannot passively diffuse through membranes and in favor of xenobiotic detoxification and excretion.

Among the three well-known xenobiotic receptors, aryl hydrocarbon receptor (AhR) is a bHLH-PAS transcription factor sharing no DNA sequence homology with the classic nuclear receptor. Its transcriptional functions require an obligatory partner ARNT. Once ligand activated, the AhR-ARNT complex interacts with the consensus DNA sequences bearing the conserved core motif (GCGTG), which is often found in the regulatory regions of the genes such as the *cyp1a1, cyp1a2* and *cyp1b1*. CAR is unique among the nuclear receptors in that its transcriptional action is constitutively activated even without ligand existed. PXR on the other hand is a bona fide nuclear receptor,

which is a ligand-dependent transcriptional regulator to regulate physiological functions involved in the xenobiotic metabolism. PXR is a unique xenobiotic receptor with its large and flexible ligand-binding pocket that enables it to bind to various structurally different xenobiotics. The known xenobiotics that interact with PXR include over 60% of clinical drugs, environmental pollutants, herbs, and microbiota metabolites, *et al*. In addition to its well characterized role in regulating xenobiotic metabolism, in recent years, it has been found that PXR plays an important role in the regulation of various physiological and pathophysiological processes such as the IBD, lipid and glucose metabolisms.

1.2 Xenobiotic receptor PXR belongs to nuclear receptor superfamily

Since the publication about researching the cloning of the first nuclear receptor-human glucocorticoid receptor (GR) in 1985[1], there has been significant progresses made in this field.

Nuclear receptor superfamily has abundant types of transcriptional regulation factors in animals. Many nuclear receptors act as ligand-regulated transcriptional regulators, and thereby connect directly between signal transduction molecules and transcription responses. This superfamily consists of receptors, such as PXR, thyroid hormone receptor (TR), retinoid acid receptor (RAR), vitamin D receptor (VDR) for regulating hydrophobic molecules like steroid hormone, thyroid hormone, retinoic acid, fatty acid, leukotriene, prostaglandins as well as lipophilic xenobiotics[2].

1.2.1 Classic structure of nuclear receptor

In terms of nuclear receptor structure, molecules like PXR, CAR and RXRα share common structural traits. Most of nuclear receptors include an N terminal ligand independent activation function 1 (AF-1), a highly conserved double zink-finger DNA binding domain (DBD), a large ligand binding domain (LBD) and a ligand dependent activation function 2 (AF-2) region at the C terminal. Nuclear receptor superfamily is usually categorized as steroid receptors, with estrogen receptor (ER) being the most investigated, orphan receptors and adopted orphan receptors such as PXR and LXR. Steroid receptors are to sense the existence of steroid hormones and act as homodimers, whereas adopted orphan receptors act as heterodimer complexes with the common nuclear receptor partner-retinoid X receptor α (RXRα). As shown in Figure 1, is the most common structure of nuclear receptor in the examples of RXR, CAR and PXRα.

However, there are many exceptions of nuclear receptors that do not follow classic nuclear receptor structure domains. For example, there are receptors that lack of DBD, such as short heterodimer partner (SHP). Our research target PXR is unique in the nuclear receptors for its larger and more flexible ligand binding pocket, making it able to sense a much wider range of the ligands than other nuclear receptors[3].


Figure 1. Functional and structural domains of PXR, CAR and RXRα as examples of nuclear receptors (A/B as AF-1, C as DBD, E/F as LBD and AF-2)[4]

1.2.2 Mode of action of nuclear receptors

Ligand-activated nuclear receptors share many common features in the mode of action. Certain nuclear receptors such as the progesterone receptor (PR), CAR are found in the cytoplasm and their nuclear translations are triggered by the ligands which are typically hydrophobic small molecules that transverse the cellular membrane by passive diffusion. After passing through the cytoplasmic membrane, the ligands interact with the nuclear receptor causing conformational changes of the receptors and releasing of the accessary proteins factors such as the heat shock proteins. Interaction with ligands seems to enhance the nuclear receptor accumulation. Interestingly, PXR has been found in both cytoplasm and nucleus. The cytoplasmic function of the PXR, however, is unknown, under investigation in our laboratory. Some receptors such as the ER has been reported to have "non-genomic" effects through regulation of no-receptor tyrosine kinases and some cell membrane proteins.

In the classic view of the nuclear receptors in the absence of agonist, the chromatin bound receptor interacts with a co-repressor complex typically containing the histone deacetylased (HDAC) at the regulatory region. Upon the agonist binding, the AF-2 region dissociates the co-repressor protein and recruits the transcriptional co-activator complexes typically containing histone acetyltransferases (HATs) as well as the mediator complexes, which in turn promote the gene expression regulated by the recruitment of the basal transcription machinery (Figure 2).

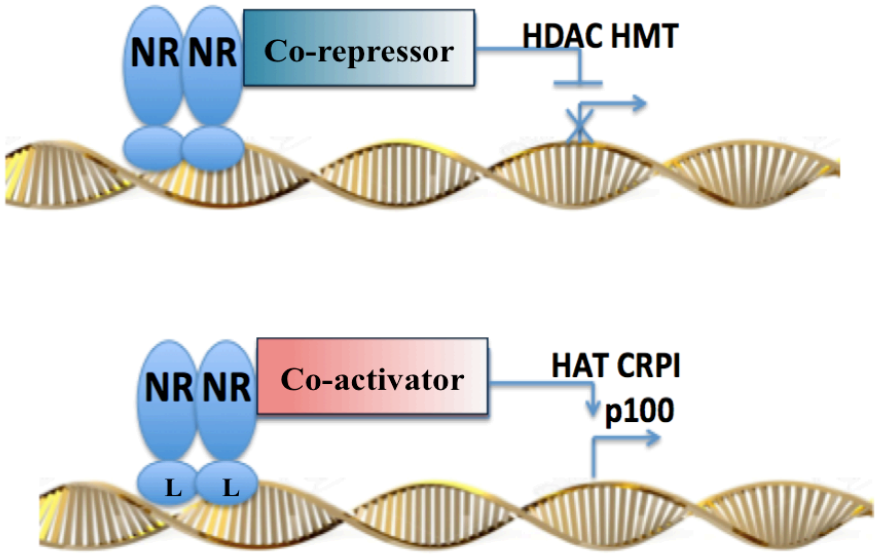


Figure 2. Transcriptional modulation of nuclear receptors

1.3 Discovery and cloning of PXR

It has been a long-standing observation that drugs such as glucocorticoid and phenobarbital induce liver enzymes such as the cytochrome P450, and the cellular

binding entity has been implicated. Searching for this binding entity and molecular characterization of these binding components is important for drug development and application. It was not till 1998, the PXR has been finally identified and characterized of the receptor at molecular level, that was made possible through the cloning and sequence identification[5, 6]. Characterization of this vital xenobiotic receptor has greatly facilitated analyzing the mechanism of the regulation of several important drug metabolizing enzymes (DMEs) and transporters. The constructions of PXR null, PXR over expressed and "humanized" PXR models have greatly facilitated the investigation of novel functions of PXR, including its regulation of innate immunity in gastrointestinal (GI) tract[7] and the maintenance of homeostasis. In addition, the cloning of the PXR made it possible to perform high throughput screening of the drug/xenobiotic for determination of their metabolism in the body.

1.4 Mode of action of the PXR regulation of gene *cyp3a4*

The mechanism of PXR regulating the gene expression involved several basic steps including binding of PXR to the ligands, nuclear translocation, association with the conserved DNA sequences (enhancer), and interaction with the co-regulators. The best-studied system is its transcriptional regulation of the cytochrome P450 (CYP) 3A4[8].

For adult human, CYP3A4 is a main DME in the body, because it predominantly expresses in liver and gut where most detoxifications take place, and also because it has a variety of substrates including clinical drugs and many endogenous substances. Regardless of the non-genetic influencing factors, the genetic effects on CYP3A4

expression are reported to be involved in the metabolism of over 60% of drug. The cytochrome P450 such as CYP3A4 regulated by PXR have been found to be highly polymorphic which may be important in causing the variations of the individual drug metabolism abilities. These variations are important for rational drug design and judicious application of the drugs.

The gene expression of the *cyp3a4* is mainly regulated by PXR, although the cross regulation between PXR and CAR has been found[9]. PXR/*cyp3a4* gene has been a prototypical regulatory model for the analysis of the mode of PXR transcription action, as shown in Figure 3.

Like most other nuclear receptors, after sensing the existence of agonists, PXR translocates into nucleus and acts with the common nuclear receptor partner, RXR α. The heterodimer complex can recruit several co-activators to bind at the XREM region, which is a regulatory region that can regulate the expression and translation, located upstream of the *cyp3a4* gene. Because of the wide range of the involvement of the PXR in both xenobiotic chemicals and endobiotic subtracts metabolisms, it is critical to understand the ligand-dependent gene regulation of PXR and its application in drug development and its clinical problems.

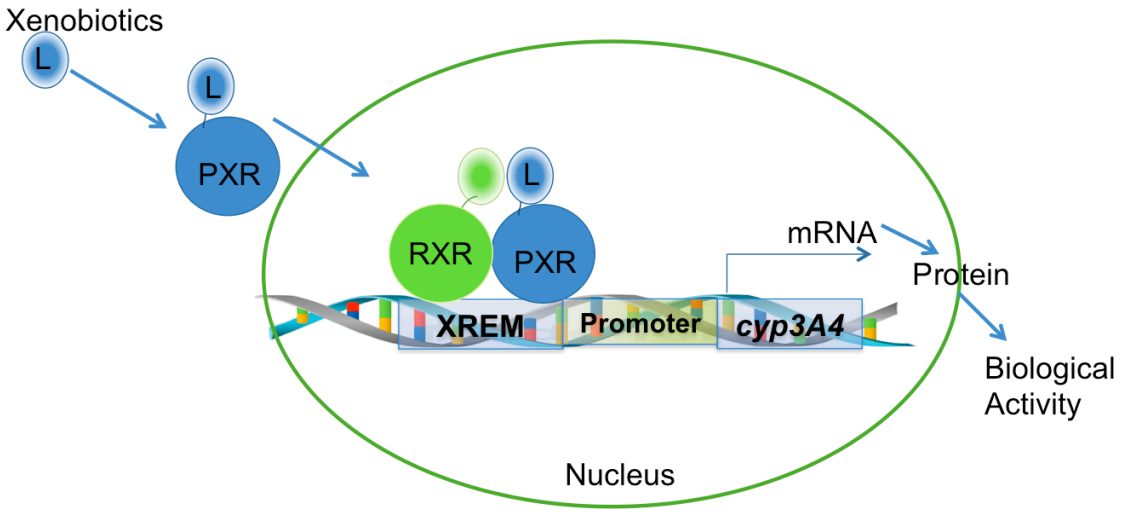


Figure 3. Mechanism of PXR action

1.4.1 The regulatory role of PXR in xenobiotic metabolism

Recently naturally occurring substances/compounds that interact with the PXR have evoked a great deal of interests. For example, the St John's Wort (SJW) has long been applied as an efficient anti-inflammatory and anti-depressant drug. Despite its effective role in the treatment of depressant, it has been found to causes drug-drug interactions (DDIs) that if using together with cyclosporine leads to enhanced metabolism and clearance of cyclosporine, and trigger adverse interactions with anti-HIV and anti-inflammatory agents if co-administered with them. Also, the indole metabolites derived from commensal microbiota such as indole 3-propionic acid (IPA) has been found as a PXR ligand, suggesting the involvement of PXR in microbiota metabolism which in turn may have important implications in drug metabolism and DDIs.

Another example indicating the importance of PXR in regulating xenobiotics is its interaction with polycyclic aromatic hydrocarbons (PAHs). PAHs are important environmental pollutants mainly deposited in fossil fuels and released when there is insufficient oxygen for complete combustion. Benzo[*a*]pyrene (BaP) is a well known PAH that is highly mutagenic and carcinogenic by forming DNA adduct, which is either detoxified or activated to its toxic metabolites in body. It has been reported that BaP is capable of the activating of CYP3A4 promoter via PXR[10]. Consistent with this observation, research from our laboratory has shown that PXR plays an important role in reducing the BaP-induced DNA damages[11]. In addition, our laboratory has found that PXR interaction with the AhR pathway through transrepression thereby reducing BaP-induced DNA damages (manuscript under review). Therefore, PXR is very important in regulating the metabolism of BaP that in favor of its detoxification pathway. The main virulence of BaP in inducing human cancer is its activation by phase I metabolizing enzymes mainly consisted of cytochrome P450 (CYP). After BaP is bioactivated to its genotoxic metabolites, it can react with the nucleophilic sites of DNA and form DNA adduct, which leads to mutation and cancer. However, the phase I metabolizing enzyme in this toxic activation pathway is also involved in the detoxification by PXR, which induces phase I/II metabolizing enzymes and transporters that in favor of the detoxification and excretion of BaP, and thus protects human body from BaP-induced DNA damage[11].

1.4.2 The regulatory role of PXR in endobiotics metabolism

Although PXR is identified as an important xenobiotic receptor, its functions in regulating endobiotics and maintaining homeostasis are equally important because it also senses and responses to various endogenous chemicals.

One family of PXR endogenous ligands is bile acid and its metabolites. Bile acid is cholesterol metabolite, which is structurally important in bile and lipid. Whereas, they are potential toxins in exceed dose, such as lithocholic acid (LCA), which is the secondary bile acid, is the cause of cholestasis. It is found that PXR can sense and detoxify LCA and thus protect from LCA-induced liver damage in experimental mice. Since experiments have proved that activated PXR is efficient in the treatment of cholestasis, PXR agonists such as rifampicin (RIF) and SJW have been successfully used as clinical drugs for cholestasis treatment[12].

In recent research, PXR has been found an important role in maintaining hormonal homeostasis and endocrine system integrity. PXR, also named as steroid and xenobiotic receptor, can sense and regulate the metabolism of hormones, especially steroid hormone, which is a regulator of various physiological activities. As it is shown in mice that genetic modified PXR (VP-PXR clone cells) and ligand-dependent activated PXR (RIF) have higher rates to activate steroidogenic enzymes to regulate steroid hormones. However, hormonal balance can be easily disrupted by xenobiotics like toxins and drugs introduced into the body, because these xenobiotics can induce DMEs and transporters that can imbalance homostasis. Because PXR acts a role as a "sensor and effector" in regulating DMEs and DDIs through its transcriptional regulation of

various DMEs and drug transporters coordinately[13], it functions as a regulator to maintain hormonal balance.

### 1.4.3 PXR involved in drug-drug interactions

PXR can be induced through a variety of ligands. Activated PXR contributes to the action of phase I/II DMEs that recognize many gene-involving drugs and substrates, that one concern about PXR-targeted drugs is their involvements in DDIs. RIF identified as an effective hPXR agonist, provides an explanation of why RIF is a PXR inducer to regulate DMEs, and RIF causes DDI in clinical use. PXR clinical activation drugs also include paclitaxel (Taxol®), the most widely used anti-tumor medicine, which is subjected to liver CYP3A4 and CYP2C8 metabolic inactivation. Besides inactivated by liver enzyme CYP450, paclitaxel can be output from intestine through the activation of PXR regulated gene (MDR1) that encodes a broad specificity proteins in intestinal efflux pump excretion system[12].

In addition, some traditional Chinese medicines (TCMs) are found involved in DDI. The biggest concern of herbal products used as clinical drugs is their side effects introduced when co-administered with other drugs. It is been found that two TCMs, Gan Cao (*Glycyrrhiza uralensis Fisch*) and Wu Wei Zi (*Schisandra chinensis Baill*) can induce PXR activity, and regulate various DMEs and transporters activations. After co-treated rat with Wu Wei Zi or Gan Cao and warfarin, which is an anticoagulant to metabolize CYP2C9, rats showed an up-regulated metabolism rate of the herbal

medicine, strengthening security defense involved in using herbs and other nutritional supplements in order to prevent PXR-mediated DDIs[12].

## 1.5 Physiological functions of PXR in intestine

PXR is a ligand-dependent transcription factor capable of regulating gene expressions that are important for xenobiotic metabolism. Besides its function as xenobiotic sensor and regulator, a novel function of PXR has been uncovered in maintaining the homeostasis of GI tract, since it has been shown that mice deficient in PXR developed IBDs, and there are intriguing interactions between PXR and intestinal barrier functions and commensal microbiota mediated by the microbial derived indole metabolites, such as IPA[7]. But in the PXR null mice, the proteins that are important for the barrier functions are down-regulated[7].

Since PXR mainly presents in liver and other parts of GI tract, its functions in xenobiotic detoxification and homeostasis maintaining are involved in many human diseases in liver and intestine[14], showing a promising future for PXR ligands application.

### 1.5.1 The anti-inflammatory functions of PXR

Xenobiotic receptors consist a chemical defense network to sense and detoxify xenobiotic chemicals and endobiotic subtracts, and the immune system conducts a biological defense. These two defense systems work independently but indispensable to each other. Coordinately, they provide a survival necessity for the wellbeing of

organisms[15]. Our laboratory has reported the interaction between PXR and nuclear factor (NF)-κB pathway, which is an important mediator in regulating the adaptive as well as the innate immune response[16]. We have observed that the activation of NF-κB by lipopolysaccharide (LPS) and tumor necrosis factor (TNF-α) resulted in the repression of PXR transcriptional activity[3]. It is reported that LPS signals through toll-like receptors (TLRs) to induce NF-κB inflammatory cytokines secretion in sepsis[17] and suppress the PXR activity, as shown in Figure 4.

Furthermore, PXR and NF-κB interaction is recently discovered to treat human inflammatory disease such as IBD, a chronic inflammation in GI tract. RIF as a typical human PXR agonist, has long been used as a immunosuppressant in suppressing immune response, and PXR is discovered to associate with the susceptibility to IBD in recent papers, since pregnenolone 16α-carbonitrile (PCN), a PXR agonist in mice, is found to protect against IBD in PXR expressed mice but has no protective function in PXR null mice.

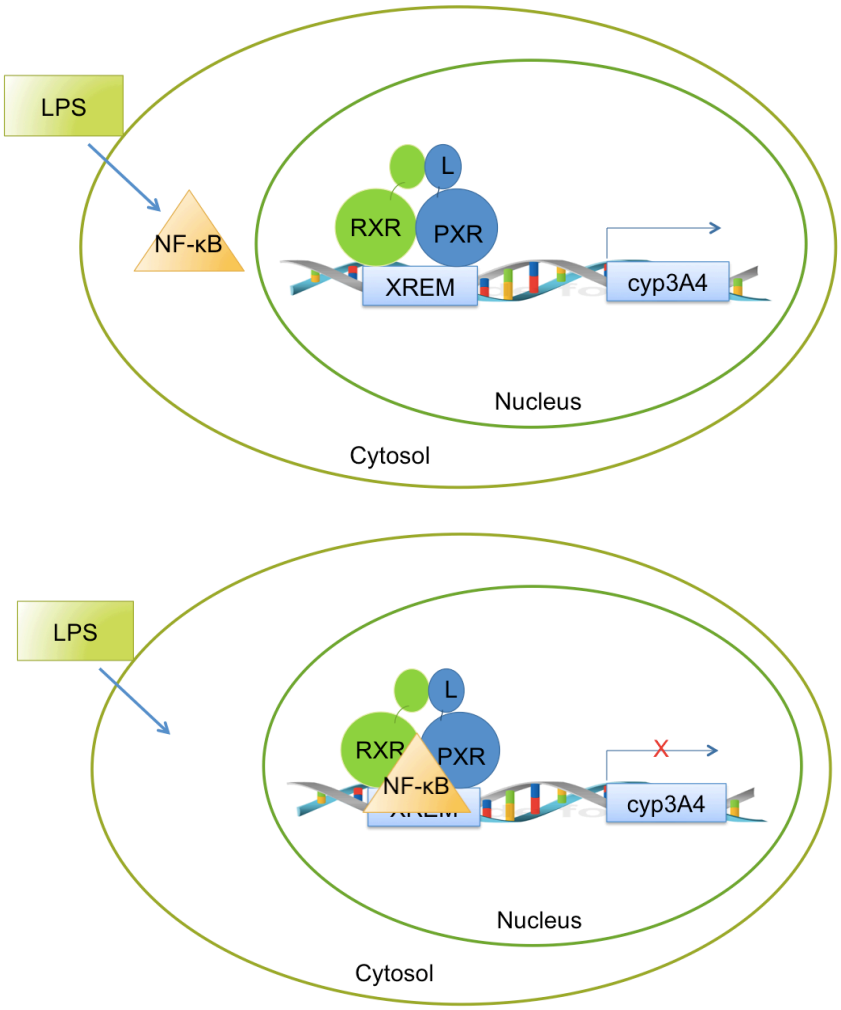


Figure 4. The mutual suppression between PXR and NF-κB

Base on these observations, we reasoned that the inflammation will cause cell susceptible to bacterial invasion because the mutual suppressive interaction of LPS and PXR. And we investigated this interaction by measuring the intercellular bacterial colony forming unit after co-treatment of LPS and PXR agonists.

### 1.5.2 PXR regulation of innate immunity

One of the recently discovered functions of the PXR besides its detoxification function is its ability to regulate innate immunity. For example, it has been found that PXR is of vital importance in protecting gastrointestinal organs from inflammatory responses induced by the harmful commensal microorganisms. In PXR knockout mice model, there are spontaneous inflammatory responses.

It has been found that PXR null mice developed spontaneous GI tract inflammation, suggesting that the importance of PXR in maintaining the homeostasis between commensal bacteria and host cells have been observed. In addition, the indole metabolites IPA derived from commensal microbiota serves as a PXR ligand in vivo, and it up-regulated mRNA expression encoded for tight junction protein. The microbiota metabolites by-product serves as a PXR ligand indicating an intricate interaction between bacteria and host cells. In this study, we proposed to mechanistically test the role of PXR in regulating bacterial resistance in the cultured colon cells.

## 1.6 *Salmonella* Typhimurium

*Salmonella* Typhimurium is an invasive pathogen that infects host cells through oral ingestion of contaminated food and water[18]. *Salmonella* Typhimurium is a very common food-borne infection and a major public health problem that causes severe diarrhea worldwide. *Salmonella* induced gastrointestinal infection is called salmonellosis[19]. Each year, *Salmonella* is estimated to cause nearly one million

illnesses in USA alone, including 20,000 hospitalizations and 400 deaths[20]. Symptoms after *Salmonella* infection including diarrhea, fever, headache and abdominal cramps after 12 to 72 hours of exposure of contaminated food or water.

Foodborne pathogens entering through the GI tract can confront several differences in the host environment, including pH, salt stress and enzymes differences. Generally, acid environment and bile acid inside GI track induce antimicrobial activity to fight against pathogenic bacterial infection. Diarrhoeal pathogens have developed several mechanisms to conquer the differences and inhospitalities of the host environment.

### 1.6.1 Mechanism of *Salmonella* Typhimurium invasion

*Salmonella* is a powerful pathogen that has developed several effective strategies to infect through the GI tract. Center of the critical virulence for *Salmonella* is the type 3 secretion system (T3SS) (Figure 5) encoded by the *Salmonella* pathogenicity island-1 (SPI-1). T3SSs are protein complexes that are responsible to translocate certain proteins directly from bacteria into the host cells. Upon contacting the surface of the host cells, the T3SS secret system is activated and secrets at least 12 effectors inside of the host cells that will mediate host cell signaling. These effectors cause membrane ruffling, bacterial invasion and tight junction disruption, although the specific functions of each effector are not identified yet[18]. Membrane ruffling and tight junction disruption finally lead to bacteria invades inside of host cells and the concomitant nuclear responses lead to the secretion of pro-inflammatory cytokines[21].

T3SS secretory system in bacteria is functioned as a protein complex that spans both inner and outer membranes. Additionally, there are components in cytoplasm that are involved in other steps of effectors secretion process including production of energy, protection of the exported protein and regulation of the bacterial invasion or secretion processes[21].

It has reported that T3SS effectors disrupt tight junction both directly and indirectly. Tight junction is located at the interface between intestinal epithelial cells including transmembrane proteins, such as claudin and occludin, which establish hemophilic interactions between adjacent epithelial cells, and plaque proteins including ZO-1 to adapt at the surface of the tight junction. Many diarrhoeal pathogens invade the epithelial barrier of the host cell by disrupting tight junction. For example, *Escherichia coli* (*E.coli*) disrupts the host cell barrier by dissociating occludin from tight junction area, and *Vibrio cholera* directly degrade the extracellular domain of occludin[18]. As for *Salmonella*, the expression of occludin is decreased after bacterial invasion[19]. In terms of infection symptoms, the break down of tight junction is believed to result in diarrhea and other important intestinal inflammation (e.g., IBD)[22]. The mechanism via T3SS of diarrhoeal pathogens infection is shown in Figure 6.

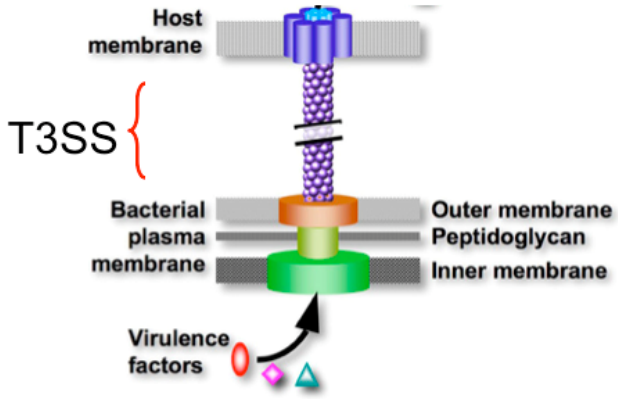


Figure 5. The type 3 secretion system[21]

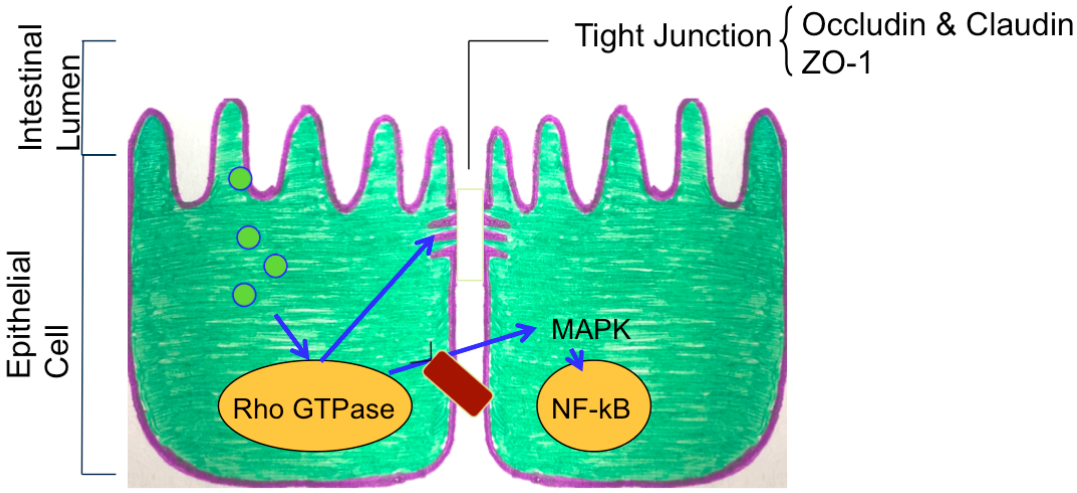


Figure 6. The mechanism via T3SS of diarrhoeal pathogens infection

1.6.2 Resistance to *Salmonella* Typhimurium invasion

An intact intestinal epithelial tight junction barrier acts as a physical block from the outside compounds to the internal environment[23].

Intestinal epithelial cells are connected by intercellular junction complexes that regulate several characteristics such as membrane polarity, integrity, and cell signaling ability. *Salmonella* is able to invade and replicate in the intestinal cells during infection. As expected, the ability of *Salmonella* to invade the epithelial cells constitutes a key step in the pathogenesis. As mentioned above, an important mechanism in host-bacteria interactions can be discovered by studying effectors that interrupt the function of tight junction[24].

It has been found PXR null mice developed spontaneous GI tract inflammation suggesting that the PXR is of vital importance in the maintenance of homeostasis between commensal bacteria and host cells. So, we investigated the regulation of PXR in the resistance to *Salmonella* infection via regulating the expression of tight junction proteins.

## 1.7 Objectives of my thesis research

One of the recently discovered functions of the PXR is its ability to regulate innate immunity. For example, it has been found that PXR is of vital importance in protecting gastrointestinal organs from inflammatory responses induced by the harmful commensal microorganisms. In PXR knockout mice model, there are spontaneous inflammatory responses in GI tract, suggesting that the importance of PXR in maintaining the homeostasis between commensal bacteria and host cells have been observed. However, it is not clear whether PXR plays a role in regulating the cellular resistance directly without the involvement of immune system. In this study, we

proposed to mechanistically test the role of PXR in regulating bacterial resistance in the cultured colon cell lines with modifications of the levels of PXR expression.

To investigate the role of PXR in regulating the *Salmonella* infection, we utilized the PXR-transfected colon cell lines in the bacterial infection assay and we found that PXP plays a critical protective role against *Salmonella* infection. We have found in an earlier study that bacterial endotoxin LPS inhibited the PXR activity, and we tested the effects of LPS of the PXR transfected cell lines and found the resistance was weakened after the cells were treated with LPS suggesting the interaction between bacterial endotoxin play a role in enhancing the infectivity of the *Salmonella* through at least in part by suppressing the PXR transcriptional activity.

Because it has been found that adopted orphan nuclear receptors are able to signaling regulate certain interactions between bacterial metabolites and host cells. For example, AhR can regulate dietary ligands, and PXR is reported an important intestinal barrier regulator. And since PXR has a large and flexible binding pocket, it might also accommodate various bacterial metabolites[22].

Since the barrier proteins are involved in the resistance of host cells to the bacterial invasion, we are interested in investigating the potential mechanism whereby PXR enhances the resistance to bacterial invasion by up-regulating the levels of expression of the barrier proteins. In our study, we investigated the possible mechanism that PXR affects the membrane protein expression level like CDC42, RAC1[25], ZO-1 and occludin[19]. The occludin is a transmembrane protein that establishes homophilic interaction between adjacent cells, and ZO-1 is an adaptor at the intracellular surface of

tight junctions to link several signaling pathways to actin cytoskeleton. These membrane proteins are the structure proteins specific related to *Salmonella* infection by affecting the stability and permeability of cells, and thus make cells more susceptible to *Salmonella* infection. So we used realtime PCR to measure the expression of these membrane proteins to investigate if there is a regulatory role of PXR in the bacterial infection.

# 2. MATERIALS AND METHODS

## 2.1 Chemical reagents

DMEM/High Glucose (HyClone, UT); Penicillin-streptomycin, phosphate-buffered saline, fetal bovine serum (Gibco); Oligonucleotides as the PCR primers (IDT); LipofectAMINE (Invitrogen, CA); Trizol (Ambion, NZ); Firely luciferase assay kit, firely luciferase lysis buffer (Biotium); All other reagents are from Sigma; iTaq universal SYBR green supermix (Bio-Rad).

## 2.2 Cell cultural

The human HepG2 and HT29 cell lines were purchased from American Type Culture Collection (ATCC, VA). Human colon carcinoma cell lines (HCT116, HCT116-PXR) were gifts from Dr. Xie (department of pharmacology and chemical biology, University of Pittsburg). Stable PXR transfected cells (PXR-HT29, PXR-HepG2) were constructed in our laboratory before.

Human colon carcinoma cells (HT29, HT29-PXR, HCT116, HCT116-PXR), and human liver carcinoma cells (HepG2, HepG2-PXR) were cultured in DMEM/high glucose with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P.S). Transfected cell HepG2-PXR need G-418 (100 μg/mL) to be specific selected at 37°C in 5% $CO_2$ atmosphere.

2.3 *Salmonella* infection assay

*Salmonella* Typhimurium (gift from Dr. Lawhon, department of veterinary pathobiology, Texas A&M University) was inoculated overnight agitated in Luria–Bertani (LB) solution at 37°C[26], because spectrophotometric results suggested overnight culture was enough time for bacteria to obtain mid-log growth phase. The bacterial concentration was around $5x10^7$ CPU/mL, and about $10^6$ bacteria were added to each well in 24-well plates.

Treated cell lines with DMSO, RIF (25 μM), hyperforin (0.2 μM), ketoconazole (25 μM) and LPS (1 μg/mL) for 24 h in 24-well plates. Plates of treated cells were inoculated with the *Salmonella*, centrifuged for 10 min at 1000 rpm and cultured for 30 min, after extensively washed the plates with phosphate-buffered saline (PBS) for three times, 5 min each time, changed the medium to DMEM/High glucose with 100 μg/mL gentamicin and cultured for 1 h. After PBS washing three times, 5 min each time, the extracellular bacteria were washed away. 0.1% Triton X-100 in PBS was applied to each well, which was then placed on an orbital shaker for 5 min to lyse the cells completely. Made a $10^4$-fold dilution of the lysed cells with PBS and bacteria were released on LB plates. Counted and calculated bacterial colony counts as CFU/mL.

2.4 RNA extraction and reverse transcription

RNA was extracted by Trizol reagent and reverse transcribed to cDNA by reverse kit. RNA purity and concentration were measured by Nanodrop ND-100 Spectrophotometer. The RNA concentration was adjusted to 1000 μg/mL.

RT-PCR reaction system was in 20 μL reaction mixtures with 12 μL of random primer (10 ng/μL), ran at 80°C for 10 min, and added 2 μL DTT, 2 μL DNTP (10 mM), 4 μL 5x first-strand buffer and 1 μL MLV enzyme from reverse transcriptase, ran at 37°C for 2 h.

2.5 Quantitative realtime PCR analysis

Realtime PCR analysis was conducted with iTaq universal SYBR green supermix two-step kit. The reversed cDNA was amplified and detected using 15 μL reaction mixtures with 7.5 μl of iTaq universal SYBR green supermix, 0.75 μL of forward and reverse primers (100 nM) respectively, 2 μL of cDNA template, and 4 μL of DEPC water. The detection system ran at 50°C for 10 min, 95°C for 2 min, along with 95°C for 10 s and 58°C for 30 s for 35 cycles. Results from housekeeping gene (β-actin) were used for normalization.

Realtime PCR primers were listed as follows: ZO-1 forward, 5'-GTGTTGTGGATACCTTGT-3', and reverse, 5'-GATGATGCCTCGTTCTAC-3'[27]; Cdc42 forward, 5′-CTTTCTTGCTTGTTGGGACT-3′, and reverse , 5′-ACACCTGCGGCTCTTCTT-3′[28]; Occludin forward, 5′-TCAGGGAATATCCACCTATCACTTCAG-3′, and reverse, 5′-CATCAGCAGCAGCCATGTACTCTTCAC-3′[29]; Rac1 forward, 5′-CCCTATCCTATCCGCAAACA-3′, and reverse, 5′-CGCACCTCAGGATACCACTT-3′[30]. The list of primer sequences and their functions is shown in Table 1.

Table 1. Primer sequences and functions in the realtime PCR assay

| Gene | Functions | Primer sequences |
|---|---|---|
| β-actin | Reference gene | AGAGCTACGAGCTGCCTGAC |
| | | AGCACTGTGTTGGCGTACAG |
| ZO-1 | Tight junction protein | F: GTGTTGTGGATACCTTGT |
| | | R: GATGATGCCTCGTTCTAC |
| Cdc42 | Membrane protein | F: CTTTCTTGCTTGTTGGGACT |
| | | R: ACACCTGCGGCTCTTCTT |
| Occludin | Tight junction protein | F: TCAGGGAATATCCACCTATCACTTCAG |
| | | R: CATCAGCAGCAGCCATGTACTCTTCAC |
| Rac1 | Membrane protein | F: CCCTATCCTATCCGCAAACA |
| | | R: CGCACCTCAGGATACCACTT |
| β-catinine | Membrane protein | F: GAACCAGACAGAAAAGCGG |
| | | R: GCTACTTGTTCTTGAGTGAAG |
| CYP3A4 | PXR regulating gene | F: CCACAAAGCTCTGTCCGA TCT |
| | | R: GAACACTGCTCGTGGTTTCACA |

2.6 Statistical analysis

Colony forming unit (CFU) was to measure and calculate viable bacteria or fungi counts. In 1 mL of bacteria solution:

$$\text{CFU} = \text{No. of colonies x dilution factor} \quad (1)$$

The results were calculated as mean ± standard deviation (SD) and applied to SPSS 20.0 to calculate the p-value. P values $<0.05$ and $<0.01$ were used to indicate statistical significance. All experiments were triplicated to make sure repeatability.

# 3. RESULTS

## 3.1 PXR stable clones and cell-based assay for determination of PXR agonist and antagonist activity

PXR stable clones were generated by stable transfection of pCI-neo plasmid carrying the hPXR cDNA in colon carcinoma cell lines HT29 and HepG2. The transfected cells were selected under the antibiotics neomycin. In addition, a luciferase reporter gene assay was developed where human PXR cDNA was stably co-transfected with CYP3A4 gene upstream regulatory regions in HepG2 cells[31]. A stable clone was chosen for the analysis of compounds with PXR against and antagonist activity. The known PXR agonists RIF and hyperforin were found to activate the luciferase reporter gene and the agonist activity could be antagonized upon co-treatment with PXR antagonists ketoconazole and clotrimazole[32] (Figure 7).

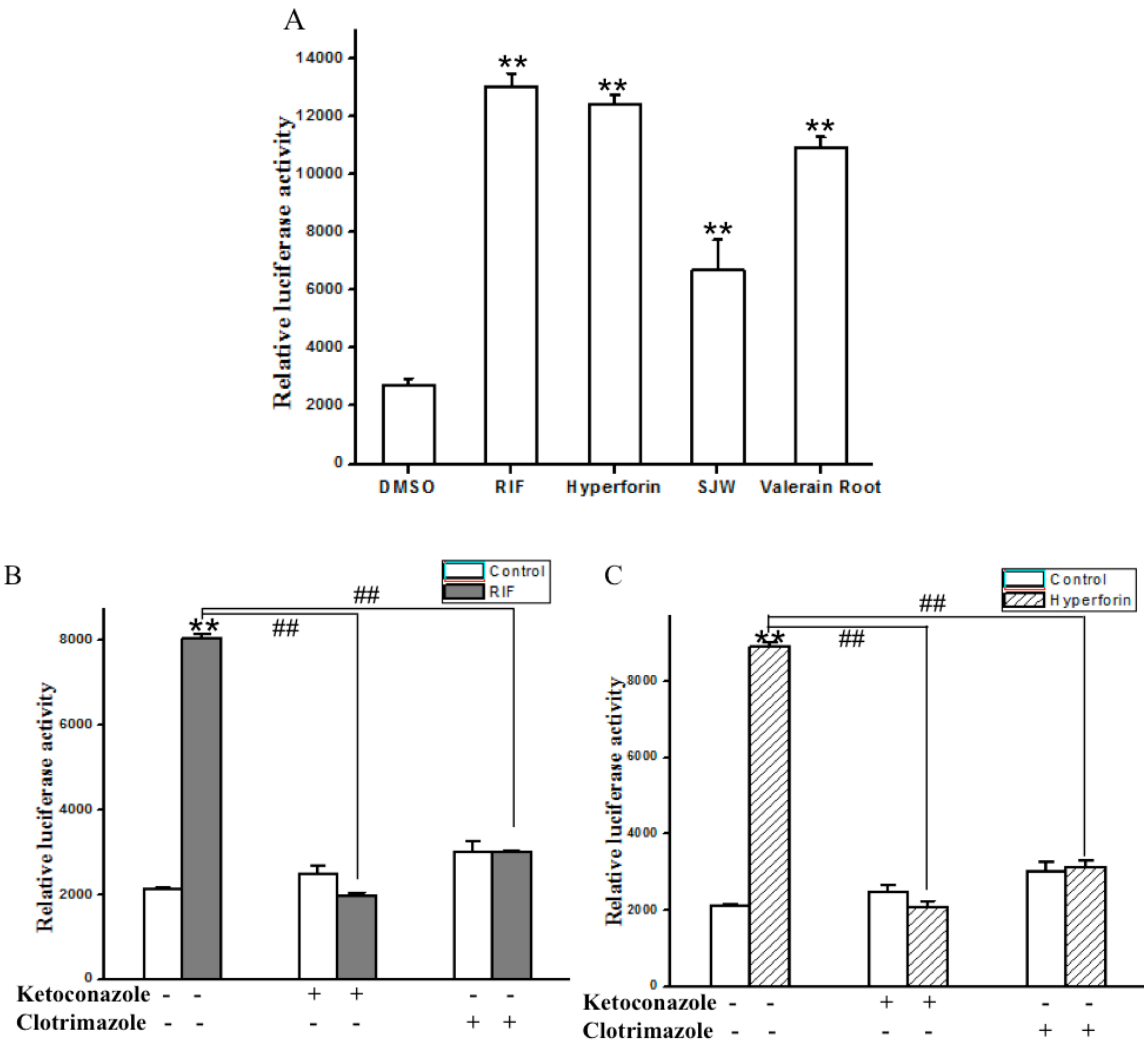


Figure 7. PXR agonist and antagonist activities determined by PXR-regulated luciferase reporter assay

A cell-based luciferase assay was performed to determine the PXR agonist and antagonist activities. A. PXR-driven luciferase reporter cell was treated with RIF (25 μM), hyperforin (0.2 μM), SJW extract (1/1000 from a one gram capsule) and valerian root extract (1/1000 from a one gram capsule) for 24 h. B. The reporter cell line was co-treated with RIF and PXR antagonists ketoconazole (25 μM) or clotrimazole (25 μM) for 24 h. C. The reporter cell line was co-treated with hyperforin and PXR antagonists ketoconazole (25 μM) or clotrimazole (25 μM) for 24 h.

PXR agonist and antagonist activities were tested in the PXR-HepG2 cell as shown in Figure 7A. It is aligned to published reports that RIF and hyperforin function

as PXR agonists and can induce PXR activity by 5 times. Additionally some herbal medicine can induce PXR activity including SJW and valerian root, but the induced rates are less than that of RIF and hyperforin. The fact that hyperforin is extracted and condensed from SJW explains that hyperforin is a more effective PXR agonist than SJW. Valerian root is most commonly used to cure sleep disorder, especially insomnia, and SJW is commonly used for depression treatment. Since PXR is discovered in many important clinical DDIs, and PXR-mediated drug interactions sometimes lead to ADIs, the uses of valerian root and SJW are suggested to avoid co-administered with other PXR-targeted drugs.

Among all PXR antagonists that inhibit PXR transactivation reported until now, the most common and investigated are the azole class of chemicals, such as ketoconazole and clotrimazole as shown in Figure 7B and 7C.

3.2 PXR regulates the resistance to *Salmonella* infection in colon cell lines

We performed the bacterial infection assay with *Salmonella* in PXR expression colon cell lines. *Salmonella* in mid-log growth phase were used to inoculate the PXR-transfected colon cell lines for 30 min and then the cells were cultured with DMEM/High glucose with 100 μg/mL gentamicin and cultured for 1 h. After PBS washing three times, 5 min each time, the extracellular bacteria were washed away. 0.1% Triton X-100 in PBS was applied into each well, which was then placed on an orbital shaker for 5 min to lyse the cells completely. Made a $10^4$-fold dilution of the lysed cells

with PBS and bacteria were released on LB plates. Counted and calculated bacterial colony counts as CFU/mL.

The colon cells expressing PXR were resistant to *Salmonella* infection in a ligand-dependent manner. Aligned with the PXR role in regulating the *Salmonella* infection, the PXR-regulated resistance could be antagonized by PXR antagonist ketoconazole (Figure 8-10).

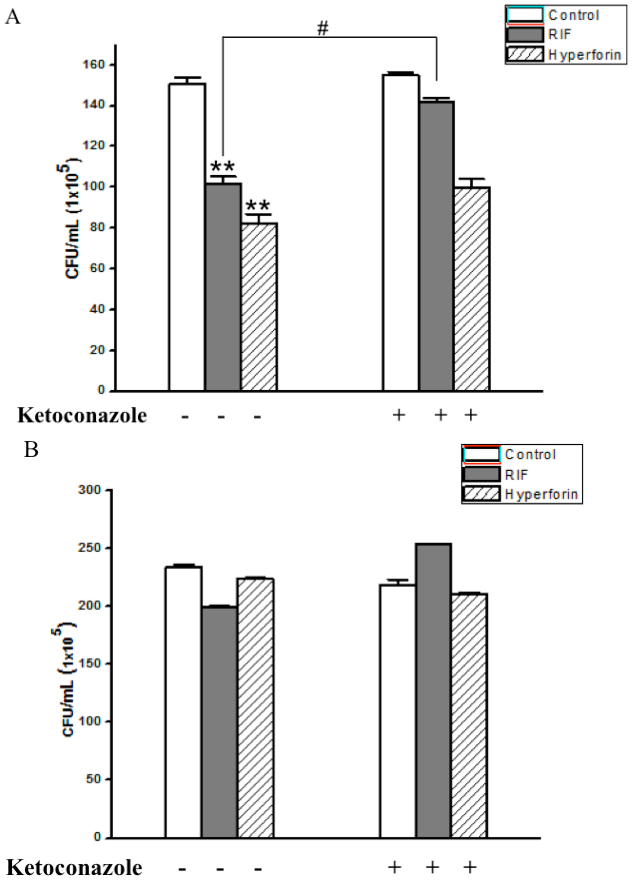


Figure 8. *Salmonella* infection assay in PXR-HT29 and HT29 cells

A is PXR-HT29 transfected cell and B is HT29 cell without PXR. The cells were pretreated with RIF (25 μM) and hyperforin (0.2 μM) for 24 h and were incubated with *Salmonella* for 30 min. The bacterial infectivity was determined by the infection assay. Antagonizing RIF- or hyperforin-induced PXR activity by ketoconazole (25 μM) resulted in increased susceptibility of host cells to bacterial infection.

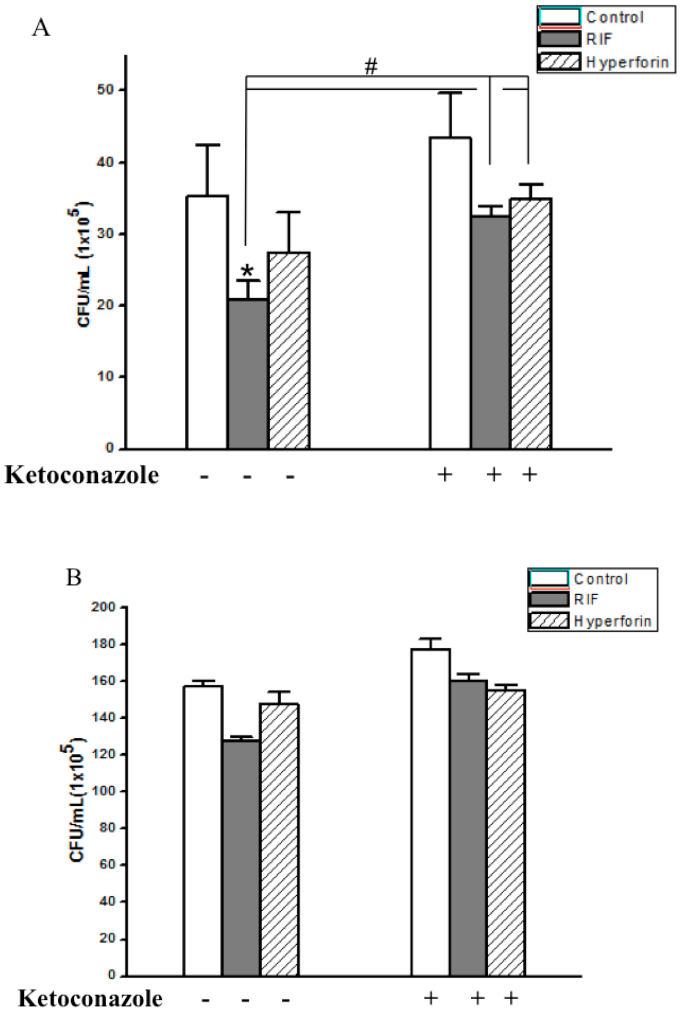


Figure 9. *Salmonella* infection assay in PXR-HCT116 and HCT116 cells

A is PXR-HCT116 transfected cell and B is HCT116 cell without PXR. The cells were pretreated with RIF (25 μM) and hyperforin (0.2 μM) for 24 h and were incubated with *Salmonella* for 30 min. The bacterial infectivity was determined by the infection assay. Antagonizing RIF- or hyperforin-induced PXR activity by ketoconazole (25 μM) resulted in increased susceptibility of host cells to bacterial infection.

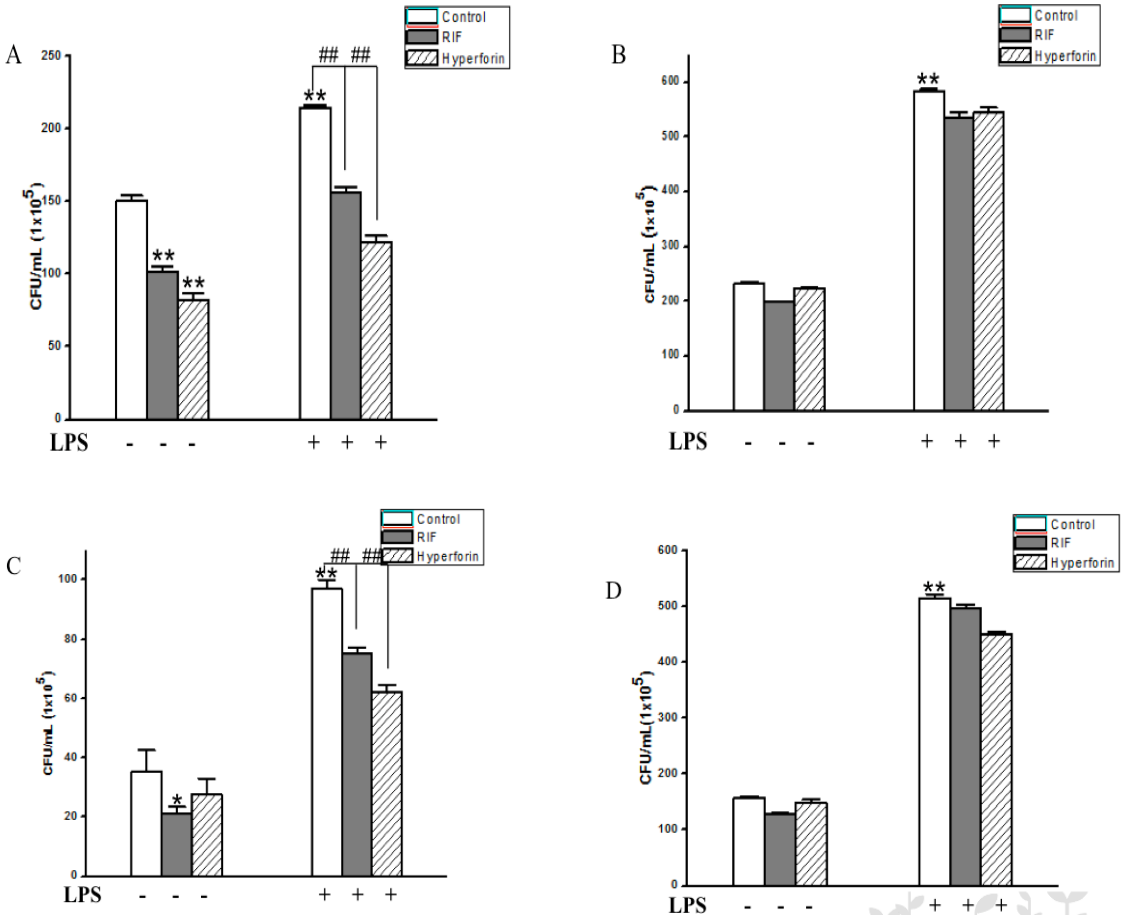


Figure 10. *Salmonella* infection assay

A and C are PXR transfected cells and D and F are cells without PXR. Cells were pretreated with RIF (25 μM) and hyperforin (0.2 μM) for 24 h and were incubated with *Salmonella* for 30 min. The bacterial infectivity was determined by the infection assay. Antagonizing PXR activity by LPS (1 μg/mL) through NF-κB pathway resulted in increased susceptibility of host cells to bacterial infection. The results showed RIF- and hyperforin-induced resistance were reversed by LPS treatment.

From Figure 8, 9A, we can see a decreased intercellular CFU count by 2 folds in PXR transfected cells but no significant decrease in HT29 and HCT116 cells, suggesting that after PXR is activated, there are less *Salmonella* invaded inside of cells. It is reported that the antagonized role of ketoconazole is functioned when co-treated with agonist, as seen in Figure 8, 9B, there are increased intercellular CFU counts in

ketoconazole & RIF and ketoconazole & hyperforin groups compared to RIF and hyperforin individually in PXR transfected cells, suggesting when PXR activation is antagonized by ketoconazole, there are increased *Salmonella* infection.

Ketoconazole is clinically used to treat fungal skin infection. Since ketoconazole does not have much antagonize function of PXR activity when working alone, people using it to treat fungi infection should not worry about the susceptibility of bacterial infection.

Interestingly, we found LPS increases *Salmonella* infection more significantly in cells lack of PXR (3 folds) than in PXR transfected cells. Even after co-treated LPS with RIF or hyperforin, intercellular bacteria only reduce slightly in cells lack of PXR in contrast with the dramatic decrease in PXR transfected cells (Figure 10). The percentage of the changing CFU is demonstrated in Table 2. These findings show that colon cells without PXR expression are more susceptible to bacterial infection, and adding PXR agonist cannot reduce intercellular bacterial counts because not enough PXR is activated in PXR deficient cells, which aligns with our earlier study that LPS activated NF-κB is mutual repressive with PXR function.

Table 2 Percentages of CFU changes in infection assay (%)

| Cell lines | RIF | Hyper | K+RIF (RIF) | K+Hyper (Hyper) | LPS | LPS+RIF (LPS) | LPS+Hyper (LPS) |
|---|---|---|---|---|---|---|---|
| PXR-HT29 | 32.4 | -45.2 | 39.3 | 21.4 | 42.6 | -27.5 | -56.2 |
| HT29 | 14.8 | -4.3 | 27.2 | 6.0 | 150 | -8.2 | -6.8 |
| PXR-HCT116 | 40.5 | -22.1 | 54.8 | 27.3 | 175 | -22.7 | -36.1 |
| HCT116 | -19 | -6.3 | 25.5 | 5.1 | 227 | -3.3 | -12.6 |

*Hyper: Hyperforin

3.3 Determination of the role of occludin in *Salmonella* infection by realtime PXR

*Salmonella* is a strong invasive pathogen that invades host cells by disrupting the host cell epithelial structure and dissociating tight junction proteins[19], among which, occludin is a transmembrane protein that establishes homophilic interaction between adjacent cells. Report showed occludin was decreased in *Salmonella* infection[19], suggesting occludin is involved in the *Salmonella* invasion process. We tested several important tight junction proteins such as occludin, ZO-1, and claudin, and found PXR acts as a regulatory role in up-regulating the expression of occludin. Figure 11 shows the expression of occludin at the mRNA level. Figure 12 shows the regulatory role of PXR in tight junction.

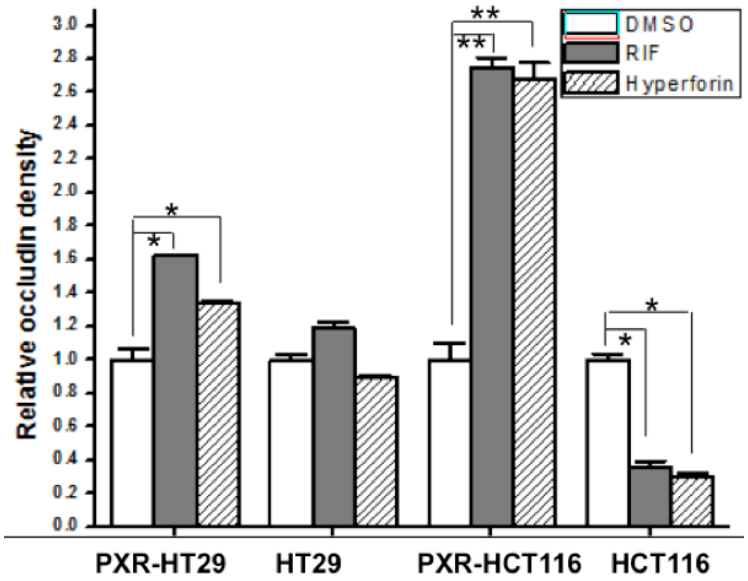


Figure 11. PXR activation results in increases in expression of barrier protein occludin
PXR-enhanced and parental cells were pretreated with agonists by RIF (25 μM) and hyperforin (0.2 μM) for 24 h, and treated cells were inoculated with bacteria for 30 min. Total RNA were extracted from the cells for realtime-PCR determination of the occludin mRNA level.

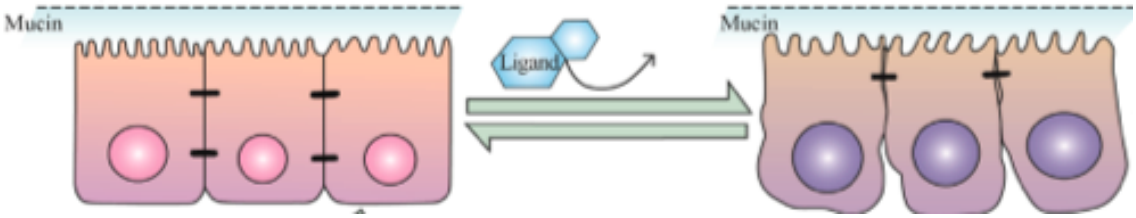


Figure 12. The regulatory role of PXR in tight junction

Occludin is a transmembrane protein that establishes homophilic interaction between adjacent cells. It is reported in *Salmonella* infection, the expression of occludin is decreased after bacteria invasion[19]. And our results shows that activated PXR can induce the expression of occludin in PXR transfected cells, but in cells without PXR, occludin expression does not change or even decrease, which aligns with our previous

study that RIF and hyperforin are harmful to cells when without PXR. The results show that PXR decreases intercellular bacterial counts by regulating the expression of occludin, which is involved in *Salmonella* invasion. Since PXR is mainly deposited in epithelial cells, it is logical that activated epithelial PXR is responsible for regulating intestinal barrier as we observed.

# 4. CONCLUSIONS

The PXR was initially identified and characterized as the xenobiotic sensor and effector[33] responsible for metabolism of various prescription drugs and xenobiotics. PXR together with other xenobiotic receptors such as AhR, CAR and FXR form a chemical defense network guarding the body against the xenobiotic insults[15] including mutagenic DNA damages induced by BaP[11]. Consistent with its role as the master xenobiotic receptor, it is highly expressed in the liver and other parts of the GI tract. PXR has been found to interact with other transcription factors such as the NF-κB[31] and this crosstalk has been found to be an important pivot for mutual regulation between the receptor-regulated pathway and NF-κB regulated immune/inflammatory pathway. The interactions between PXR and NF-κB have been found to be important for the maintenance of the homeostasis of GI tract as the PXR null mice demonstrated spontaneous intestinal inflammation[7]. Clearly, the complexity of the GI tract microbiota with their metabolites harbor enormous potentials for mediating the interactions between microbiota and host cells, and PXR with its wide range of ligand specificity is likely to play a critical role in regulating the host-microbiota interaction as demonstrated in several recent studies[7]. To understand the role of PXR in regulating the interactions between pathogen and host GI tract, we analyzed the effects of PXR on the invasion of *Salmonella* in colon cell lines transfected with PXR.

Many cancer cell lines from the GI tract such as HepG2 and HT29 have reduced the level of PXR expression, and the reduced PXR expression has been suggested as part

of the pathogenesis. Indeed, it has been shown the pediatric Chron's disease is associated with the reduced PXR expression, and in mice activation of PXR has been found to reduced inflammation and inhibited tumorigenesis[34, 35]. One of the underpinning mechanisms for the diseases is postulated to be the crosstalk between PXR and NF-κB.

Results from recent studies have revealed novel functions of PXR in regulating the various physiological functions including the immune responses, lipid and glucose levels, and homeostasis of GI tract through its ability to interact with the natural metabolites from the intestinal microbiota[7]. Recently, accumulating results suggest that deficiency in PXR are linked with the inflammation in the GI tract[36]. However, the mechanisms involved in the protective functions are likely to be multiple dimensions, one of which is its role in regulation of immunity against bacterial infection. The interactions between commensal microbiota and intestinal epithelium are important for the homeostasis of the GI tract and of particular interest is a recent report indicating that symbiotic bacterial metabolites regulate gastrointestinal barrier function via the xenobiotic sensor PXR, one may surmise PXR may play a role in guarding the GI epithelium against the invasion of the pathogenic bacteria. We utilized human colon cell lines with transfected PXR to analyze the potential role of PXR in protecting the cells from *Salmonella* invasion and also investigated the role of PXR in regulating the expression of the barrier genes such as occludin, a transmembrane protein located at tight junction. Our results strongly suggest that PXR plays a pivotal role in the epithelium against the bacterial invasion.

In summary, PXR shows a protective role to resist *Salmonella* infection in PXR transfected cells. One mechanism is by regulating the expression of a tight junction protein-occludin.

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